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Differentiation of the Vegetative and Sporogenous Phases of the Actinomycetes

1. The Lipid Nature of the Outer Wall of the Aerial Mycelium

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SUMMARY: The characteristic dry powdery appearance of the aerial mycelium of actinomycetes and the difficulty of wetting the constituent spores appear to be due to lipid substances in their outer walls. These substances are removed by fat solvents, and wetting agents, destroyed by alkali and are probably glyceride in nature. A staining with Sudan IV in ethanol clearly distinguishes the lipid-containing aerial mycelium from the vegetative mycelium.

The somewhat greater diameter of the aerial spore-bearing filaments of the ordinary aerobic actinomycete (the *Streptomyces* of Waksman, Group I *Cohnistreptothrix* of Ørskov) as compared with that of the vegetative portion of the mycelium has long been recognized (Ørskov, 1923). The marked resistance of the aerial spores to desiccation was noted by Acosta (1895) and Berestneff (1907) who established viability after 9 and 10 years' drying, respectively. More recently von Plotho (1940), as the result of various micro-chemical and tinctorial tests, stated that 'die Actinomyceten ihre Sporen-membranen mit Substanzen ausrüsten, die den Hyphenmembranen fehlen', but drew no definite conclusions as to the nature of these substances. In current reports on the production of antibiotic agents from stationary liquid cultures of the actinomycetes, there is frequent mention of the need for optimum yield of active substance, of a good surface growth (bearing aerial mycelium). The use of mass transfers of spores to obtain an early surface growth, and the failure to do so with an inoculum of mainly vegetative sub-merged colonies, are well known to all workers in this field. Nevertheless, the cause of the flotation of the spores and the sinking of the main mycelium has not hitherto been investigated. That the spores are strongly hydrophobic is evident from the common observation that a drop of water added to an old plate bearing a continuous sporing surface growth can be rolled round the dish without losing its shape, acquiring only an external coating of spores. This phenomenon did not escape the acute observation of Ørskov (1923), who recommended adding a drop of ethanol under the coverslip when examining colonies with an aerial mycelium 'since the aerial mycelium, which partly shuns water, will by this means be drawn down' (p. 39). An attempt has been made to relate this hydrophobe property to the lipid nature of the outer layer of the aerial filaments.

Organisms tested

Two characteristically pigmented varieties were extensively tested: (1) a member of the *Actinomyces coelicolor* species-group with its insoluble red, soluble blue, indicator pigment, having spirally coiled aerial hyphae with oval

to spherical spores on most common media; (2) a member of the *A. griseofulvus* species-group, producing no soluble pigment, with fawn to greenish straight aerial hyphae and cylindrical spores. Both were isolated from soil.

In addition some 100 strains picked haphazardly from routine soil platings were tested with fat stains. They represented a considerable number of varieties, including several members of the chromogenous *A. albus* type producing a melanin on protein-containing media, *A. flavus, aureus, flavovirens, cellulosae,* etc. No attempt was made to give specific names to all the strains. The random method of selection ensured a varied sample of the soil population.

Two or three common penicillia and aspergilli, and some saprophytic strains of mycobacteria, sarcinae, streptococci, and coliform bacteria were used for comparison.

Fat stains

(*a*) *Sudan IV* (see Conn, *Biological Stains,* 1946). None of the methods commonly employed for demonstrating fatty substances *within* cells was suitable for this external sheath of fatty material. As already noted by Lewis (1941) dried fixed films are very unsatisfactory for the general run of fat-containing bacteria. The usual solvents—mixtures of ethanol and acetone—are unstable and precipitate the dye overmuch on the slide, though the technique of Burdon, Stokes & Kimbrough (1942), where an emulsion of cells is made in the solution of the dye (in their case Sudan black B) and a loopful rapidly spread with a circular motion on the slide, ensures that the precipitated particles are deposited at the periphery of the drop. Using this method with a 70 % solution of Sudan IV in ethanol, a pink colour was achieved around clumps of 4-month-old spores; 3-day-old spores of the same strain did not stain; *Mycobacterium phlei,* in fairly dense masses, and a suspension of finely ground beeswax were positive; *Bacterium coli* and *Chromobacterium prodigiosum* were negative. But in all cases the pink stain was dull and faint compared with the brilliant red given by a drop of cotton-seed oil. *Iso*propanol, as recommended by Lillie (1944), improved the intensity and penetration of the stain, but the best results, with no precipitation of the dye, were obtained with *n*-butanol.

The following technique was elaborated. Actinomycetes are grown on the surface of sterile cellophan over agar of different compositions. Portions of the growth are removed entire by cutting sections of the cellophan at varying intervals. It is possible thus to obtain the whole of the substratum mycelium of a colony, which otherwise would be adherent to the agar, together with the delicately attached aerial mycelium *in situ,* and to preserve both intact on the cellophan throughout the staining operations.

A 70 % butanol staining solution is prepared as follows: A, stock saturated solution; add 0·5 g. Sudan IV (B.D.H. 627701/44083) to 25 ml. *n*-butanol; boil (117°), cool and filter. B, mix 4·5 parts *n*-butanol with 5·5 parts by volume of ethanol. For the staining solution, add 7 volumes of A to 9 volumes of B and filter. This solution remains stable for months.

The cellophan bearing the growth is stained for 30 min., dipped for a few seconds into 70 % ethanol to remove excess stain, washed in water, and mounted either in water for immediate examination or permanently in glycerol jelly.

In all strains tested the vegetative substratum mycelium remained entirely colourless, while the aerial filaments even before division into conidia showed varying degrees of external staining. Day-old, minute branches can be distinguished by the incrustation of red granules. Mature spores in long chains are evenly stained round each member (cf. Pl. 1, fig. 1). Precise definition is obtained only if the growth is not too dense (growth 5–10 days old, according to the nature of the medium, being suitable); the individual filaments of thick tufts of sporulating branches, characteristically situated on the crown of an older colony, tend to be stuck together with the dye, giving a blurred though abundantly stained picture. On the other hand, isolated spores mechanically disrupted from the branches are often unstained. Nevertheless, the validity of the technique has been generally confirmed by sampling aerial mycelium from various slope cultures on different media, spreading it out on cellophan, and staining as above, whereby the majority of the spores still adhering in chains of varying length will take the stain. Surface sporing colonies on liquid media require more care in handling and must be dried before the staining schedule is applied, but a proportion give positive results. Coverslips pressed on soil and sand cultures for a few days, then fixed in *n*-butanol and stained, in some cases, retain conidial branches in a sufficiently undamaged state to take the Sudan stain (Pl. 1, fig. 2).

The composition of the medium, therefore, appears to have no influence upon the production of the lipid substance or substances in the walls of the aerial mycelium. The point was further tested by growing the two chief test organisms on cellophan strips over a considerable variety of synthetic agars suitable for actinomycetes. The same differential staining of aerial and substratum mycelium was found, although naturally more vivid and consistent pictures were obtained on media such as starch tryptone agar which enhance sporulation.

As long as the aerial branches remain unbruised and unwetted, there is little diminution in staining capacity with ageing of the culture. Portions of growth on cellophan removed from the agar and kept in the dry state for over a year, and the surface growth on soil plate cultures allowed to dry out over the period of one year, have retained their staining properties.

No positive results have been obtained by any method with the bottom vegetative growths of actinomycetes in liquid cultures; with streptococci, a chain of which resembles in gross appearance an actinomycetal chain of conidia; or with any of the ordinary rod-shaped bacteria, cocci, or sarcinae tested. The dye aggregates loosely round sporing heads of aspergilli and penicillia, which, like those of actinomycetes, are also difficult to wet. Partial staining of the lipids in the acid-fast mycobacteria, which has frequently been described, was confirmed.

(*b*) *Osmic acid.* The walls of the spores but not the membranes of the vegetative hyphae were stained a characteristic yellow-brown when specimens were inverted for 2–4 days over a cell containing a drop of 2 % osmic acid in a moist chamber.

(c) *Oil blue N.A.* This dye (1, 4-*bis*-amylaminoanthraquinone; Calco., American Cyanamid Co.), which is used for staining rubber as well as fatty substances, acts rapidly either in the 70 % *n*-butanol solution or in 60 % *iso*propanol (cf. Lillie, 1945), producing in 5–10 min. a blue aerial and a colourless substratum mycelium, which after washing are dramatically distinguishable to the naked eye in the maturer colonies. Microscopically the picture is more blurred by dye precipitates than in comparable Sudan IV-stained material. A lactophenol solution prepared according to Wittenberger (1944) is stable, and if allowed to act for 24–48 hr., the material being subsequently washed in 25 % lactic acid, gives a more even stain.

Extraction with fat solvents

Portions of 7-day-old growth on cellophan over Czapek agar were extracted in small covered containers at 33° for 24 hr. with the following fat solvents: acetone, ether, chloroform, xylene, benzene, ethanol, *n*-propanol, *iso*propanol, *tert.*-butanol, and *n*-butanol. They were then dried and stained by Sudan IV. In all cases excepting the *n*-butanol treated specimen, the staining of the aerial mycelium was very much less than in untreated material. The *n*-butanol extracted growth, though brilliantly red to the naked eye, showed on microscopical examination a rather fuzzy outline as compared with the control, probably due to the enhanced adsorption of the dye. Acetone, ether, and benzene removed almost all the stainable material round the spores, which were a faint yellowish pink. Chloroform and xylene were nearly as effective. Ethanol yielded a poorly stained picture with a considerable amount of dye precipitate. Somewhat pinker spores were left in the material treated by the propanols and by the *tert.*-butanol. In all instances the spore membrane, even if only faintly coloured, appeared to be thicker than the cell walls of the substratum mycelium.

The efficacy of the acetone and ether treatments suggests (a) that the bulk at least of the fatty material is not phospholipin in nature, and (b) that it is not very firmly bound in the cell wall. An attempt was therefore made to extract the substance by a simple modification of Anderson's (1932) methods for the lipids of tubercle bacilli. The 5-day-old, abundantly sporing cellophan growth of fifty plates was submitted to ether-ethanol-chloroform extraction in the cold. The final chloroform extract on evaporation *in vacuo* yielded a minute quantity of an orange-coloured, waxy, semi-fluid substance. The amount was too small for detailed tests, though it proved to be glyceride in nature. Cotton-wool fibres rubbed into the substance retained the wax in small agglomerates which coloured a brilliant red with Sudan IV, and which darkened on exposure to osmic acid. It is, of course, impossible to prove that part at least of this substance was not extracted from the interior of the cells, but it is worth noting that Lieske (1921) found that with most actinomycetes the old dried cultures, which would probably be abundantly sporing, yielded a small quantity of ethereal extract 'der scheinbar auf Fette zurückzuführen' (p. 88).

Saponification

Portions of 7-day-old growth on cellophan placed in ethanolic KOH in covered glass vessels lost almost every vestige of stainable material within 3 hr. at 33°. Similar material sealed in a cell with a few drops of ammonia and KOH according to the method of Molisch (1923) yielded after 2 days some crystals, presumably of soap, as well as amorphous protrusions round the aerial filaments. Other portions of cellophan growth placed in 10 % ammonia were found to show a yellowing and apparent thickening of the spore membrane, frequently in bipolar fashion, which was visible on the 2nd day of treatment, obvious from the 6–12th days, thereafter degenerating into irregular swellings.

Action of wetting agents

Large single colonies, one month old, were treated for 3 days in 1 % soap solution, and also in three different commercial detergents. When washed, dried, and stained with oil blue N.A., they were conspicuously unstained in contrast to colonies taken from the same plate, which were either untreated or dipped in *n*-butanol. Three-day-old growth of the same strain, when immersed in one of the detergents (solution A, a sulphonated long-chain ester), was wetted almost instantaneously and sank to the bottom of the container in less than 5 min. At this period it still took up the fat stains, but less vividly than the control. Untreated growth which has already taken the stain is not decolorized by immersion for prolonged periods in the wetting agents. Untreated spores ride in a layer on the surface of an aqueous suspension and extend in a film up the walls of the tube, the greater part remaining there even after 5–10 min. centrifugation at 10,000 r.p.m. This property is to a very considerable extent lost if the spores are first washed off from the growth in detergent A instead of in water; the majority go into suspension and deposit on standing.

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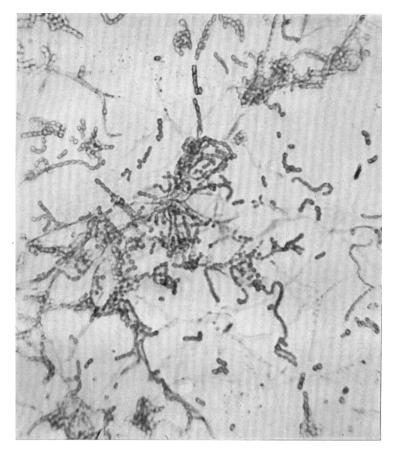
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EXPLANATION OF PLATE
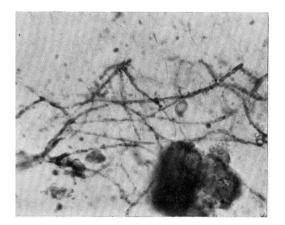
Fig. 1. Mature spores stained with Sudan IV, substratum mycelium colourless; cellophan growth on agar 8 days.

Fig. 2. Aerial mycelium not divided into spores, stained with Sudan IV, substratum mycelium colourless; impression preparation from soil culture 4 days.

Fig. 1

Fig. 2

D. ERIKSON—LIPIDS IN ACTINOMYCETHS. PLATE 1